Troponin I Tyrosine Phosphorylation Decreases Myofilament Ca^{2+} Sensitivity and Accelerates Deactivation

Undergraduate Research Thesis

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by

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ABSTRACT

The post-translational modification of troponin by phosphorylation is a central mechanism to modulate calcium activated cardiac contraction. Over 95% of phosphorylation in the heart occurs on serine or threonine residues. It has been recently shown that phosphorylation of the troponin inhibitory subunit (TnI) at Tyr-26 is decreased in human heart failure with functional effects such as decreasing thin filament calcium sensitivity and accelerating calcium dissociation. This discovery has lead us to hypothesize that the phosphorylation of the other tyrosine residues in TnI, including TnI Tyr-29, Tyr-112 (and TnI Tyr-134 found mouse cardiac muscle), will also exhibit functional effects. Kinase prediction calculation suggests that these additional tyrosine residues are even more likely to be phosphorylated than Tyr-26. To test the regulatory effects of TnI Tyr-phosphorylation, we generated, expressed and purified cardiac TnI containing phosphomimetic substitutions (Tyr to Glu or Tyr to Asp). Purified proteins were used to form the troponin complex and functional effects determined by measuring calcium binding to Troponin C (TnC) in reconstituted thin filaments. Our results confirm Tyr-26 pseudo-phosphorylation decrease calcium binding to TnC on the thin filament and demonstrate a similar decrease in calcium binding to TnC with the pseudo-phosphorylation of Tyr-29 in thin filaments containing human cardiac TnI and thin filaments containing mouse cardiac Tyr- 134 TnI. To investigate the effects of TnI Tyr phosphorylation on myofilament deactivation, we measured the rate of calcium dissociation from TnC. Results demonstrate filaments containing Tyr-26 and Tyr- 29 pseudo-phosphorylated TnI accelerate the rate of calcium dissociation from TnC. Based

upon these results we conclude that the pseudo-phosphorylation of TnI at these additional Tyr-residues play a functional role in modulating force production of the heart.

Chapter 1- Background

General Heart Function

The heart is the central pump of the circulatory system. During the cardiac cycle, the heart cyclically contracts and relaxes, providing a continuous circulation of blood, nutrients and oxygen throughout the body, as well as the continuous removal of wastes from the body organs. Performance of the heart is measured as the cardiac output (CO), which is dependent on the heart rate and stroke volume. Stroke volume is the amount of blood ejected by the left ventricle in one contraction. Physiological and pathological changes in body blood demand cause compensatory alterations of the cardiac muscle contraction and the blood pumped to maintain necessary blood (nutrients, oxygen and waste removal). In addition to contraction performance of the heart to change the volume of blood pumped, cardiac output is also dependent on the heart rate. After contraction there is a period of cardiac diastole, where the heart refills with blood due to relaxation of the cardiac muscle. Relaxation must also be altered to maintain an adequate volume of blood pumped by the heart. Increased heart rate requires quicker contraction and relaxation. Upon increased heart rate the hearts' ability to fill (eg. lusitrophy-the relaxation of the heart muscle) is important to maintain CO. If the muscle is unable to adequately relax, the heart will not fully fill before the next contraction cycle resulting in less blood volume being pumped at the next contraction. Therefore the modulation of both cardiac contraction and relaxation are critical to match the heart's supply of blood to the body.

There are many causes of heart disease but heart disease is ultimately the inability of the heart to pump adequate blood to meet the bodies demand for oxygen and/or nutrients. Cardiovascular disease (CVD) is the leading global and US cause of death, accounting for more than 17.3 million deaths per year [1]. There is currently no treatment for heart disease except heart transplant, and current therapeutics are ineffective. Reversing the negative changes that occur in heart failure and/or improving heart contractile function allowing heart to meet the body's demand could be beneficial. One mechanism to improve heart contractile function would be to design new therapies, however we must first understand the basic mechanism's responsible for the physiological and pathological changes in heart contraction. Conducting research to continue to learn and understand the functions of the heart will allow us to gain a closer insight on heart disease and potential possibilities to reverse or exploit these changes to modify the effects.

Regulation of Cardiac Contraction: Excitation-Contracting Coupling

The heart is a muscle composed of myocytes consisting of myofibril bundles containing myofilaments. These myofibrils have distinct, repeating units known as sarcomeres, which are the basic contractile units of the myocyte. The sarcomere is composed of thick and thin filaments –myosin and actin. The length of the sarcomere can be altered by physiological "sliding" interactions between actin and myosin [2]. The sliding of actin over myosin causes the sarcomere length to shorten, contraction of the cell and the subsequent production of force. The process of myocyte contraction occurs through a series of events called excitation-contraction coupling. Excitation-contraction

coupling is the process where an action potential triggers a cardiac myocyte to contract [2]. The thin filament associated regulatory proteins are responsible for regulating actin and myosin interactions, and thus force production. There are three states of the thin filament that regulate the actin-myosin cross bridge; blocked, closed and open [3-5]. In the blocked state the myosin-binding site on actin is sterically blocked by tropomyosin, in the closed state the myosin-head is bound weakly to the actin and in the open state the myosin head is strongly bound to actin and able to undergo the force producing power-stroke motion sliding actin to shorten the sarcomere. This state of the thin filament is regulated by calcium (the signal molecule), which controls the contraction and relaxation of the chambers of the heart. In the absence of Ca^{2+}, the TF resides in the "blocked" state. Upon stimulus to contract, an action potential depolarizes a myocyte that causes a small amount of calcium ions to rush into the myocyte through L-type calcium channels. These calcium ions trigger the release of sarcoplasmic reticulum-stored calcium through the calcium-release channel known as the Ryanodine receptor during a process called calcium-induced-calcium-release. Intracellular calcium concentration significantly increases, and free calcium subsequently binds to the troponin complex at the myofilament. The binding of Ca^{2+} to troponin causes a conformational change, initiating the movement of tropomyosin from its blocking position on actin to the "closed" state of the thin filament. A myosin-binding site on the actin is exposed so that the myosin head weakly binds and the strong binding of myosin induces the "open" state. In the open state, the myosin ATPase results in ATP hydrolyses that supplies energy for a conformational change in the actin-myosin complex powerstroke. The powerstroke of myosin displaces actin, occurring in a sliding motion, shortening the sarcomere.

Contraction occurs when the filaments slide over one another in a series of repetitive myosin powerstroke events. As long as there is sufficient intracellular calcium such that the calcium remains bound to TnC, the thin filament remains in the "open" state and myosin cycling continues.

Relaxation occurs after the end of this contraction phase when an ATP-dependent calcium pump, sarcoplasmic/endoplasmic reticulum calcium ATPase, moves calcium back to the sarcoplasmic reticulum and a sodium-calcium-exchange pump transports the remaining extra calcium out of the muscle cell simultaneously. The intracellular calcium concentration is now lowered and calcium is released from troponin, inducing a conformational change in the troponin complex, and tropomyosin resumes a position on actin to block the myosin-actin binding site.

Detailed mechanism of Troponin regulation

The thin filament consists primarily of the protein actin, which contains a myosin-binding site, where the myosin molecule has the ability to bind its head. Tropomyosin molecules contribute to blocking these active sites during muscle relaxation in addition to the troponin protein molecules. Troponin is a vital protein switch that affects heart contraction and relaxation through binding the calcium signal. Troponin (Tn) is a complex of three regulatory proteins (troponin C, the calcium binding subunit; troponin I, the inhibitory subunit; and troponin T, the tropomyosin binding subunit). Troponin T binds to tropomyosin, interlocking them to form a complex. In the absence of calcium, the TnI C-terminal inhibitory peptide binds actin, contributing to an actin-myosin inhibition. Upon increased intracellular calcium beyond TnC affinity, calcium ions bind

to TnC to produce conformational changes in TnC that are relayed to the entire troponin complex. Calcium mediated changes in the TnC N-terminus expose a hydrophobic "sticky patch" that binds the TnI C-terminal switch peptide removing TnI-actin inhibitory binding [1]. With the removal of TnI and changes in TnT, tropomyosin movement is allowed and actin-myosin interaction contraction is ensured. Contraction inhibition is achieved by reverse of this process.

Troponin I Modulation in the regulation of contraction/force

With each beat of the heart, every cardiomyocyte contracts. These myocytes are not fully activated with every contraction. In the heart muscle you can therefore increase contraction by activating more Tn resulting in increased myosin binding to actin. This is in opposition to skeletal muscle where the individual skeletal muscle cells and troponin molecules are always fully activated each time the cell contracts. The degree of cardiac contraction is determined by the force (number of myosin molecules interacting with actin) and rate (cycling of these myosin cross-bridges) of the heart muscle. While many mechanisms exist to modulate cardiac contraction, changes in calcium binding to Tn are a primary mechanism of contractile modulation. The post-translational modification of Tn by phosphorylation is a significant physiological/pathological mechanism to modulate the interaction of actin and myosin and therefore contraction. These phosphorylations can result in changes in both force and rate by altering calcium binding to TnC and the amount of thin filament activation. Phosphorylation of Tn characterizes a primary mechanism to alter the responsiveness of troponin to calcium and modulate calcium-dependent thin filament regulation of myosin's interaction with actin and consequently the force of cardiac muscle.

The best-known mechanism of cardiac contractile modulation occurs through beta-adrenergic receptor (BAR) stimulation and phosphorylation of Tn; specifically at TnI serines 23 and 24 [6-7]. Unless otherwise noted, the reference to all TnI amino acid numbers is derived from the human TnI sequence. TnI, the inhibitory subunit of the troponin complex can be phosphorylated at multiple sites [8], which is a key regulatory mechanism to alter the calcium regulation of contraction via sensitivity (the affinity of calcium to binding to TnC of the troponin complex and the resulting force production of the myocyte upon binding). Upon BAR stimulation, the activation of protein kinase A (PKA) results in the phosphorylation of TnI at Ser-23 and Ser-24 that through changing Tn structure and function, results in altered TF regulation to modulate contractile function [9]. This results in decreased calcium sensitivity (which is a decrease in calcium affinity of binding to TnC) resulting in decreased contraction [10]. As calcium sensitivity decreases, it takes more calcium to attain the same contractile force production and provide the necessary volume of blood to the body because of the lower affinity for calcium to bind to the TnC-calcium complex resulting in less Tn molecules activated. Decreased calcium sensitivity leads to less Tn being activated, and therefore, less myosin molecules that can bind actin ultimately causing less force production. In addition, TnI phosphorylation results in accelerated calcium dissociation from the TnC-calcium complex (a lower affinity for calcium to stay bound). As calcium dissociation is increased, relaxation of the heart muscle is achieved more quickly, allowing the muscle to fully expand and fill with blood to the necessary volume for normal heart pumping. This affects the hearts' performance in responding to environmental and internal changes to the body.

TnI Phosphorylation at Novel Tyr-26 residue

It has recently been shown that a novel phosphorylation of TnI occurs in the human heart at tyrosine 26 (TnI Tyr-26) [12]. This is the only myofilament tyrosine phosphorylation to be identified to occur. Zhang and colleagues further showed this novel TnI Tyr-26 phosphorylation is reduced by ~ 43% in the failing human heart and models suggesting a role during cardiac pathology [12]. We have further determined the effects of TnI Tyr-26 phosphorylation on cardiac muscle. We demonstrated phosphate incorporation and pseudo-phosphorylation at TnI Tyr-26 decreases Ca^{2+}-sensitive development of force and thin filament activation as well as that TnI Tyr-26 pseudo-phosphorylation accelerated thin filament Ca^{2+} dissociation [13]. We propose that by decreasing Ca^{2+}-sensitivity, the heart's ability to function properly will be diminished because the heart will need more calcium than normally present to contract at a force capable of supplying proper blood to the body. Alternately, relaxing at a hastened rate, the heart will expand faster, filling with more blood and pumping more blood during the next contraction phase.

Tyrosine phosphorylation has not been extensively studied as a direct mechanism to modulate cardiac contraction. In addition, there are no demonstrations of myofilament tyrosine phosphorylation to alter heart function or tyrosine signaling by separate kinases from Ser/ Thr. TnI tyrosine phosphorylation therefore represents a novel phosphorylation pathway to differentially modulate cardiac contractile function through Ca^{2+} sensitivity and thin filament deactivation.

Kinase prediction software predicts that kinases are as likely, if not more likely, to phosphorylate the additional human tyrosine residues, Tyr-29, Tyr-112 and mouse Tyr-134, of TnI than the Tyr-26 site. The discovery of the functional effects of TnI Tyr-26 phosphorylation has lead us to hypothesize that the phosphorylation of additional sites in the TnI inhibitory subunit, including Tyr-29, Tyr-112 and Tyr-134 will also exhibit functional effects on cardiac contraction. In human, Tyr-133 is a non-phosphorylatable phenylalanine (Phe, F) while the corresponding mouse TnI residue is a tyrosine (Tyr-134). Due to a different number of amino acids in humans, F133 is the same TnI residue as mouse Y134. Our 2nd hypothesis proposes that the presence of the non-phosphorylatable phenylalanine residue in human TnI, in place of the phosphorylatable Tyr in mouse TnI, results in a loss of the Tyr-134 regulatory site found in mouse cardiac muscle.

Phosphorylation sites and classical Ser/Thr signaling pathways of cardiac TnI, such as TnI Ser 23/24, have important physiological and patho-physiological cardiac functions. Unlike these residues, cardiac TnI tyrosine phosphorylation has not been extensively studied. In addition, soluble tyrosine kinase signaling is not as prevalent and has not to date been demonstrated as a regulatory signaling pathway to directly alter cardiac contraction. We therefore propose that TnI tyrosine residue phosphorylation represents a novel phosphorylation pathway to differentially modulate cardiac contractile function through Ca^{2+} sensitivity and thin filament deactivation.

Chapter 2- Methods

cDNA constructs

All cardiac TnI residue numbers presented in this manuscript are given according to the native human sequence including the first methionine.

Site-directed mutagenesis (QuickChange Lightning, Agilent, Santa Clara, CA) was conducted according to manufacturer's instructions as previously described [10] to generate cDNA constructs encoding human and mouse TnI pseudo-phosphorylations: Tyr-26 to Glu (Y26E), Tyr-26 to Asp (Y26D), Tyr-29 to Glu (Y29E), Tyr-134 to Glu (Y134E). All resultant constructs were verified by DNA sequencing.

Protein expression and purification

Plasmids encoding the individual recombinant human cardiac Tn subunits were transformed, expressed in *Escherichia coli* and purified to homogeneity as previously described [3, 6, 7]. $TnC^{C35S,T53C,C84S}$ was labeled with 2-(4'-iodoacetamidoanilo)naphthalene-6-sulfonic acid (IAANS) as previously described [8, 9]. Cardiac Tn complexes were prepared by reconstitution of the individual subunits and sequential dialysis as previously described [6]. Rabbit skeletal actin and bovine cardiac tropomyosin were prepared from acetone powder as previously described [10-13]. Thin filaments were reconstituted as previously described [7].

Thin filament steady-state Ca^{2+} binding to TnC

Steady-state fluorescence measurements were conducted using a Perkin-Elmer LS55 spectrofluorimeter. IAANS fluorescence changes of labeled TnC in reconstituted thin filaments upon addition of various Ca^{2+} were monitored as previously described [10, 13]. Briefly, fluorescence was monitored from reconstituted thin filaments containing various TnI mutations in buffer (in mM; 150 KCl, 3 $MgCl_2$, 2 EGTA, 200 MOPS, pH 7.0) at 15°C with constant stirring. Calcium sensitivities of conformational changes are reported as the dissociation constant (Kd), representing a mean of titrations.

Ca2+ dissociation from TnC in the thin filament

Ca^{2+} dissociation from TnC in thin filaments containing wild type (WT) or mutant TnI was measured in calcium saturated buffer (in mM; 150 KCl, 3 $MgCl_2$, 0.2 Ca^{2+}, 10 MOPS, pH 7.0) upon rapid mixing with EGTA buffer at 15°C in a stopped flow instrument (Applied Photophysics Ltd. model SX.18 MV) with a dead time of 1.4 ms as previously described [10, 14] and kinetic values obtained. Katherine Baruk generated and made the thin filaments for these measurements and all calcium dissociation measurements presented in this thesis were collected by Shane Walton in Dr. Jonathan Davis's laboratory at The Ohio State University.

Data processing and statistical analysis

Data are presented as mean ± the standard error of the mean. Normalized steady-state Ca^{2+} binding values were fit with a logistic sigmoid function mathematically equivalent to the Hill equation to determine 50% maximal binding as previously described [6, 11]. Kinetic values of Ca^{2+} dissociation were fit using a program (by P. J.

King, Applied Photophysics Ltd.) utilizing the nonlinear Levenberg-Marquardt algorithm [14]. Results of steady-state Ca^{2+} binding and Ca^{2+} dissociation were compared by 1-way ANOVA with Tukey's post-hoc test. $P<0.05$ was considered statistically significant.

Chapter 3- Effects of Human TnI Tyr Phosphorylation

Introduction

The heart pumps blood containing nutrients and oxygen systemically throughout the body as a result of the contraction and force development of the cardiac muscle cells. The magnitude of cardiac contraction and its rate of relaxation are influenced by intracellular Ca^{2+} binding to the troponin complex to activate the thin filament allowing myosin's interaction with actin. Calcium binding to troponin (Tn) is a significant component of thin filament regulation of the actin myosin interaction and therefore, heart contraction. Phosphorylation of the cardiac troponin inhibitory subunit (TnI) modulates thin filament activation and cardiac contraction. Troponin I residue Tyr-26 has previously been identified as a novel TnI phosphorylation residue in the heart [12]. Recent work has identified that TnI Tyr-26 phosphorylation is present in the normal human heart and its phosphorylation is decreased during heart failure [12]. Our lab demonstrated that both the incorporation of phosphate and pseudo-phosphorylation at the TnI Tyr-26 residue decreases and the Ca^{2+}-sensitive development of force as well as accelerates thin filament Ca^{2+} dissociation [13]. The human cardiac TnI subunit has two additional tyrosine residues, including Tyr-29 and Tyr-112. Kinase prediction software suggests that these additional TnI Tyr residues are even more likely to be phosphorylated in the human heart than Tyr-26. We hypothesized that the phosphorylation of these sites will also alter calcium sensitivity and calcium dissociation from the troponin complex in the thin filament. To test the regulatory effects of these other TnI tyrosine phosphorylations, we generated, expressed and purified human cardiac TnI containing phosphomimetic

substitutions (Tyr to Glu or Tyr to Asp). Purified proteins were used to form the troponin complex and functional effects determined by measuring steady-state calcium binding to and dissociation from troponin C (TnC) in reconstituted thin filaments. We found that pseudo-phosphorylation of human cardiac TnI at residues Tyr-26 and Tyr-29 decreases calcium sensitivity and accelerates calcium dissociation. These identified contractile effects of the TnI Tyr phosphorylations suggest that the phosphorylation of these sites may be significant to modulate contractile function and present a novel pathway to modulate heart function.

Methods

See chapter 2.

Results

Our lab previously demonstrated that the phosphorylation of TnI at residue Tyr-26 decreased thin filament calcium sensitivity and accelerated calcium dissociation [13]. We further hypothesized that the phosphorylation of the additional Tyr-29 residue of TnI will also exhibit similar functional effects. To investigate the functional properties elicited by TnI Tyr-29 we first needed to construct, express and purify human wild-type troponin (hTnWT) and pseudo-phosphorylated TnI, mimicking phosphate incorporation. The cDNA clones encoding pseudo-phosphorylated TnI consisting of human cardiac TnI with Tyr-26 mutated to Glu (hTnY26E), human cardiac TnI With Tyr-26 mutated to Asp (hTnY26D), and human cardiac TnI with Tyr 29 mutated to Glu (hTnY29E) were generated by PCR. Resulting TnI clones and the other troponin subunits were expressed

in *E. Coli*. The resulting troponin subunits and associated thin filament proteins were then purified to homogeneity as demonstrated in figure 1. Next we determine the effect of hTnWT on Ca^{2+} regulation. Thin filaments were reconstituted with actin, tropomyosin and hTnWT and thin filament activation measured as the change in IAANS fluorescence upon steady-state Ca^{2+} binding to TnC. Consistent with our labs' previous studies, the thin filaments containing hTnWT exhibited a pCA_{50} of 5.8 +/- 0.037, n=14 (Fig 2) [13].

We next sought to verify the functional effects of hTnY26 pseudo-phosphorylation. We previously demonstrated the incorporation of phosphorylate at hTnY26E decreases the calcium-sensitive force production of cardiac muscle [13]. Performing steady-state calcium-binding experiments in reconstituted thin filament we confirmed that the hTnY26E and hTnY26D both decrease calcium binding to TnC similarly to which was seen previously by our lab. Results demonstrate thin filaments containing hTnY26D exhibited a pCa_{50} of 5.476 +/- 0.0551, n=3 and hTnY26E also exhibited a pCa_{50} of 5.476 +/- 0.0263, n=6. Comparing these values to the pCa_{50} of hTnWT (pCa_{50} = 5.802 +/- 0.037; Fig 2), we see that the pseudo-phosphorylation of Tyr-26 statistically decreases calcium sensitivity in the thin filament by +0.326 +/- 0.0107 and +0.326+/- 0.0181 pCa_{50} units, respectively for hTnY26E and hTnY26D (Fig 2) [13]. Thus, our results demonstrate we were able to confirm previous measurements for hTnWT, hTnY26E and hTnWT.

To investigate the effect of the additional phosphorylatable tyrosine residue site hTnY29 on Ca^{2+} regulation, the change in IAANS fluorescence upon steady-state Ca^{2+} binding to TnC in the reconstituted thin filament was measured for human cardiac TnI containing pseudo-phosphorylated Tyr-29 mutated to Glu (hTnY29E). Thin filaments

containing the hTnY29E exhibited a pCa_{50} value of 5.4775 +/-0.02312, n=3 (Fig 2). This value is nearly identical to the pCa_{50} exhibited by hTnY26D and hTnY26E demonstrating that the phosphorylation of these sites decrease calcium sensitivity of the thin filament to a similar degree.

Additionally, we looked at the dissociation rate of calcium from TnC of each pseudo-phosphorylated TnI in the isolated troponin [13,15]. We observed that the Ca^{2+} dissociation, measured as the average change by IAANS fluorescence by stopped-flow, from TnC in isolated troponin containing WT or pseudo-phosphorylated human cardiac TnI exhibited no significant decrease in the rate of calcium-dissociation from isolated troponin (rate of calcium dissociation: hTnWT = 42.27 +/-0.75 /s, hTnY26D = 45.11 +/-2.99 /s, hTnY26E = 42.85/s +/-1.38, hTnY29E = 43.87+/-2.87 /s; $p>0.05$; Fig 3). However, when thin filaments were reconstituted with Tn containing pseudo-phosphorylated TnI Y26 or Y29 we observed the rate of Ca^{2+} dissociation from TnC in the thin filament was significantly accelerated when compared to those thin filaments containing hTnWT (rate of hTnWT= 108.77 +/-1.19 /s, hTnY26D = 403.6 +/-22.23 /s, hTnY26E = 353.49 +/-14.43 /s, hTnY29E = 297.18 +/-21.02 /s; $p<0.05$; Fig 4). This data demonstrates that the pseudo-phosphorylation of TnI Tyr residues in the thin filament dramatically accelerates calcium dissociation from the thin filament and suggests when these TnI tyrosine residues are phosphorylated in the cardiac muscle they will accelerate cardiac muscle relaxation.

Discussion

This study aimed to confirm the contractile regulatory properties elicited by the novel N-terminal phosphorylation of TnI at Tyr-26 recently identified in the heart [11,12] and further assess the functional properties elicited by phosphorylation of the additional human TnI Tyr-29 residue. Findings of this study validate previous data from our lab [12]. In addition, major findings demonstrate that hTnY29E exhibited decreased calcium sensitivity similarly to hTnY26E in addition to similarly accelerated calcium dissociate rate. These findings support the significance of TnI Tyr-29 phosphorylation to increase thin filament deactivation, and in turn, may have an importance for increased cardiac relaxation. We propose that TnI Tyr-29 phosphorylation is beneficial during exercise for it has the ability to increase the heart rate due to its increase in heart relaxation, allowing the heart to get ready to contract more quickly. TnI Tyr-26 phosphorylation is associated with contractile modulation during heart failure [12]. Tyr-26 phosphorylation was shown to be decreased in the failing heart and that this increase may potentially contribute to the increased calcium sensitivity during heart failure [27]. Additionally, we propose that the decrease in the phosphorylation of TnI Tyr-29 may be good in heart failure because a decrease of its presence will obtain an increase in force production from contraction of the heart. The presence of inherently phosphorylated TnI Tyr-26 suggests it plays a role in maintaining normal heart function [12]. Our findings suggest the presence of TnI Tyr-29 phosphorylation may also play a role in normal and/or pathological heart function.

Chapter 4: Mouse Cardiac TnI Tyr-26 and Tyr-134 Phosphorylation Alters Function

Introduction

Contraction and force development of the cardiac muscle cells' determine the volume of blood containing nutrients and oxygen that the heart pumps systemically throughout the body. Intracellular calcium levels influence these processes. Calcium binding to troponin (Tn) is a significant component of thin filament regulation of the actin myosin interaction and therefore, heart contraction. Human tyrosine residue 26 in the inhibitory subunit of the cardiac troponin complex troponin I (TnI) has previously been identified as novel phosphorylated residue in TnI and recent work has determined that TnI Tyr-26 phosphorylation is present in the normal human heart and is decreased during heart failure [12]. Our lab has demonstrated that the incorporation of phosphate or pseudo-phosphorylation at TnI Tyr-26 decreases the Ca^{2+}-sensitive development of force and thin filament activation as well as accelerates thin filament Ca^{2+} dissociation [12]. The mouse cardiac TnI subunit has an additional potential tyrosine phosphorylation site at Tyr-134 that through differential evolutionarily selection resulted in the human TnI sequence consisting of a non-phosphorylatable phenylalanine residue (Phe-133). Human cardiac TnI Phe-133 is the same residue as mouse cardiac Tyr-134 due to mouse TnI containing the addition of an alanine residue at position 25 that is not present in the human TnI. We hypothesized that calcium binding in thin filaments containing pseudo-phosphorylated mouse cardiac TnI with Tyr-134 mutated to Glu (mTnY134E) will have a functional effect similar to that of mTnY27E. To test the regulatory effects of these other

mouse TnI Tyr-phosphorylations, we generated, expressed and purified cardiac TnI containing phosphomimetic substitutions (Tyr to Glu). Purified proteins were used to form the troponin complex and functional effects determined by measuring calcium binding and dissociation from the troponin C (TnC) subunit in reconstituted thin filaments. We found that mTnY134E decreases calcium sensitivity and accelerates calcium dissociation. These identified contractile effects of the Tyr-134 phosphorylation suggest that the phosphorylation of the TnI Tyr-134 residue may be significant to modulate contractile function and present a novel pathway to modulate heart function in the mouse heart that is not present in the human heart.

Methods

See chapter two.

Results

TnI tyrosine phosphorylation is a novel mechanism to alter heart function. We have demonstrated the functional effects of human TnI pseudo-phosphorylation of Tyr-26. It is important to compare the effects of TnI Tyr-26 across species to allow the interpretation of results found in rodent models to human cardiac function. To test the species effects of TnI on thin filament regulation, we generated, expressed and purified mouse, rat and human cardiac troponin subunits (Figs 1, 5 and 6). We investigated the effect of species troponin isoforms on calcium sensitivity by measuring calcium binding to TnC in the thin filament. Comparing human wild-type TnI (hTnWT) to the mouse wild-type TnI (mTnWT) we observed that the mouse cardiac TnI exhibits a similar

calcium-binding sensitivity to that of the human cardiac TnI ($pCa_{50;}$ hTnWT= 5.802 +/- 0.037, mTnWT= 5.870 +/- 0.0478, n=19; $p<0.05$; Fig 7). Interestingly, when we measured the steady-state calcium binding of thin filaments containing wild-type rat cardiac TnI (rTnWT) we observed the rTnWT exhibited a calcium sensitivity that was significantly decreased compared to both human and mouse (Fig 7). This decreased Ca^{2+}-sensitive binding to TnC in the reconstituted thin filament containing rTnWT suggest that amino acid differences in the Tn complex between species are important in calcium sensitivity. These WT values provide critical insight to understanding the basic difference between species calcium sensitivity that are important in future studies by providing a basis to compare the functional effects of inter-species pseudo-phosphorylations on calcium sensitivity.

In addition, we characterized the rate of Ca^{2+} dissociation from TnC of each species in the reconstituted thin filament in order to compare cardiac muscle relaxation kinetics. Stopped-flow fluorimetry was used to monitor the change in fluorescence of IAANS-labeled TnC in Ca^{2+} saturated thin filaments following rapid mixing with EGTA in order to access the rate of Ca^{2+} dissociation from TnC in the thin filament. Results demonstrate that thin filaments containing mTnWT exhibited the quickest Ca^{2+} dissociation from TnC followed by filaments containing hTnWT, while the slowest were thin filaments containing rTnWT (rate of calcium dissociation; mTnWT = 175.75 +/- 8.79 /s, hTnWT = 108.77 +/-1.19 /s, rTnWT = 103.03 +/-4.08 /s; $p>0.05$; Fig 8, 9). These values are an important factor in the determination of how filaments containing phosphorylated tyrosine sites of TnI from different species affect cardiac muscle

relaxation. This data tells us that mouse cardiac relaxation happens much quicker than both human and rat cardiac relaxation.

Similar to that in the human cardiac TnI, mouse cardiac TnI has a tyrosine-27 residue capable of phosphorylation. Mouse cardiac TnI residue 27 is identical to human cardiac TnI residue 26 because mouse cardiac TnI contains an extra amino acid at reside 25. We hypothesized the phosphorylation of this site will behave similarly to the phosphorylation of human TnI Tyr residues 26. To test the regulatory effects of TnI Tyr-phosphorylation, we generated, expressed and purified mouse cardiac TnI containing phosphomimetic substitutions (Tyr to Glu or Tyr to Asp) (Fig 1). Purified proteins were used to form the troponin complex and functional effects determined by measuring calcium binding to TnC in reconstituted thin filaments. The effect of mouse cardiac Tn containing pseudo-phosphorylated TnI on Ca^{2+} regulation was determined by measuring the change in IAANS fluorescence upon steady-state Ca^{2+} binding to TnC in the reconstituted thin filament. The thin filaments containing mouse pseudo-phosphorylated TnI Tyr-27 to Glu (mTnY27E) exhibited a pCa_{50} of 5.69+/- 0.0380, n=7. Compared to the pCa_{50} exhibited by mTnWT, which was 5.870+/-.04783, hus, the mTnY27E pseudo-phosphorylation decreases calcium binding to TnC in the reconstituted thin filament (Fig 10). This decrease in calcium sensitivity observed in the mouse cardiac TnI upon tyrosine 27 phosphorylation is parallel to the decrease in calcium sensitivity observed between hTnWT and hTnY26E. The change in pCa_{50} from hTnWT to hTnY26E is 0.326 (Fig 2). The change in pCa_{50} from mTnWT to mTnY27E is 0.18 (Fig 10). Thus, the functional effect of Tyr-26/27 phosphorylation is observed to similar degrees across species.

The mouse TnI contains an additional Tyr residue that is not present in the human TnI that kinase prediction suggests may be an even better site for phosphorylation. This site, Tyr-134 in mouse cardiac Tn is a Phe-133 in human cardiac Tn. We hypothesized that the loss of this tyrosine residue in human TnI may be evolutionarily significant. To test this we generated, expressed and purified mouse cardiac Tn containing pseudo-phosphorylated TnI Tyr-134 to Glu (mTnY134E) for steady-state Ca^{2+} binding experimentation.

The effect of thin filaments containing mTnY134E exhibited a pCa_{50} of 5.652 +/- 0.0288, n=14 (Fig 10). This decrease in calcium sensitivity is nearly identical to the pCa_{50} exhibited by mTnY27E, suggesting the phosphorylation of these sites decrease calcium sensitivity of the thin filament to a similar degree (Figs 11 and 12). Additionally, the effect of tyrosine phosphorylation observed in both mouse cardiac and human cardiac TnI appears to be proportional across species. Thus, our lab proposes that the phosphorylation of mouse cardiac Tn Tyr-27 and Tyr-134 is beneficial to heart function upon increased heart rate.

We also looked at the dissociation of Ca^{2+} from TnC of each pseudo-phosphorylation in isolated troponin and in the reconstituted thin filament in order to compare cardiac muscle relaxation kinetics [12,17]. The average change in IAANS fluorescence of stopped-flow Ca^{2+} dissociation from troponin containing mouse TnI (mTnWT, mTnY26E, mTnY134E) pseudo-phosphorylation shows no significant influence on calcium-dissociation from isolated troponin alone (rate of calcium dissociation: mTnWT = 36.75 +/- 0.85 /s, mTnY26E = 38.29 +/-0.68 /s, mTnY134E = 34.41 +/-0.84 /s; $p>0.05$; Fig 13). Additionally, reconstituted thin filaments containing

the varied pseudo-phosphorylated mouse cardiac TnI also did not differ in calcium dissociation form TnC. (rate of calcium dissociation: mTnWT = 175.75 +/- 8.79 /s, mTnY134E = 191.32 +/-14.93 /s; $p>0.05$; Figs 9 and 11). This set of data demonstrates there is a difference in calcium dissociation rates amongst species. However, unlike the effects of human TnI that was significantly faster in the presence of tyrosine pseudo-phosphorylation, the rate of calcium dissociation from thin filaments containing mouse TnI Y134E pseudo-phosphorylation was not different from that of the non-phosphorylated mouse TnI. These findings suggest that the pseudo-phosphorylation of TnI Tyr-134 has no effect on the relaxation of the mouse heart.

Discussion

In this study we demonstrate the following findings: 1) That mTnY27E exhibits a decrease in calcium sensitivity compared to mTnWT that is proportional in size to the decrease in calcium sensitivity seen in hTnY26E compared to hTnWT. 2) Thin filaments containing mTnY134E exhibit decreased calcium sensitivity compared to filaments containing mTnWT. 3) Thin filaments containing mTnY134E do not alter calcium dissociation.

The first goal of this study aimed to assess the functional properties elicited by the phosphorylation of cardiac TnI in different species to provide a basis for comparison of pseudo-phosphorylation effects across species. Findings showed that the pseudo-phosphorylation of mTnY26E has proportional functional effects as the pseudo-phosphorylation of hTnY26E, decreasing calcium sensitivity binding similarly. These

results suggest that we can use the mouse as a model in future studies to compare functional effects of human tyrosine phosphorylation in TnI.

The second major finding demonstrated that the pseudo-phosphorylation of mTnY134E exhibited decreased calcium sensitivity similarly to the pseudo-phosphorylation of mTnY27E while not altering the rate of calcium dissociate. The findings give the first demonstration of mTnTyr-134 function and therefore may have an importance to alter cardiac function. Based upon these findings we suggest the effect of Tyr-134 phosphorylation to decrease calcium sensitivity will increase the force of cardiac contraction. Furthermore, since Tyr-134 phosphorylation does not affect calcium dissociation this increase in force production would occur without impairment of cardiac relaxation. Overall we proposed a decrease of TnI Tyr-134 phosphorylation would be beneficial in heart failure to increase contraction without impairment of relaxation.

Chapter 5- Discussion

This study aimed to confirm the functional properties elicited by the phosphorylation of cardiac TnI Tyr residues [12,13], and further assess the functional properties elicited by the additional phosphorylation of TnI at Tyr-29 and Tyr-134. Findings of the study validate previous studies conducted by our lab. In addition, major findings demonstrate: 1) Mouse and human cardiac TnI have similar functional properties in the reconstituted thin filament. 2) Human TnI Tyr-26 and Tyr-29 pseudo-phosphorylation decreases Ca^{2+}-sensitive thin filament activation and accelerates thin filament deactivation. 3) Mouse TnI Tyr-27 and Tyr-134 pseudo-phosphorylation decreases Ca^{2+}-sensitive thin filament activation. 4) Mouse TnI Tyr-27 pseudo-phosphorylation decreases Ca^{2+}-sensitive thin filament activation to a similar degree as that observed in human TnI. 5) Mouse TnI Tyr-134 pseudo-phosphorylation does not alter thin filament calcium dissociation.

Regulatory effects of TnI Tyr-26 exemplify the first known myofilament protein tyrosine phosphorylation to directly modulate cardiac function, playing a significant role in contractile regulation of the heart. Previously demonstrated by our lab, the Tyr-26 pseudo-phosphorylation mutation is sufficient to recapitulate Tyr-26 phosphate effects on Ca^{2+} sensitivity [12]. Therefore, for these experiments, pseudo-phosphorylation mutations were used. Additionally, our lab has also demonstrated that a non-phosphorylatable amino acid modification of the Tyr-26 residue to phenylalanine did not alter sensitivity and therefore, the effects of desensitization come from the negative charge at the residue [12]. This is important because knowing there is no effect on desensitization of the thin filament in the presence of a neutral charge at the residue; we can assume our findings

employing pseudo-phosphorylated TnI mimics the structure/function of TnI containing phosphate at this residue.

Troponin I Tyr-26 phosphorylation has been demonstrated to be decreased in the failing human heart [12] and its functional effects are associated in with contractile modulation during heart failure [12]. The decrease in TnI Tyr-26 phosphorylation in heart failure may therefore contribute to the increased sensitivity of Ca^{2+} during heart failure and increased cardiac contraction. In addition, the presence of basally phosphorylated TnI Tyr-26 suggests it plays a role in maintaining normal heart function [12]. Our findings that TnI Tyr-29 phosphorylation also decreases calcium sensitivity and accelerates calcium dissociation suggest this phosphorylation may also play a role in the physiological modulation of normal heart function [13]. Furthermore, a decrease of TnI Tyr-29 phosphorylation during heart failure may therefore also be beneficial to improve cardiac contraction in heart failure.

We first found that thin filaments containing the mouse cardiac TnI exhibit a similar calcium-binding sensitivity to that of filaments containing the human cardiac TnI. Additionally, steady state calcium binding of thin filaments containing wild-type rat cardiac TnI exhibited a calcium sensitivity that was significantly decreased compared to both human and mouse (Fig 7,14). This decreased calcium sensitive binding to TnC in the reconstituted thin filament containing wild-type rat cardiac TnI suggest that amino acid difference in the troponin (Tn) complex between species are important in calcium sensitivity. These WT values will be important in future studies for they provide critical insight to understanding the basic difference between species calcium sensitivity and suggest that each species must be independently validated. Overall, these WT values

provide a basis to begin to compare the functional effects of inter-species pseudo-phosphorylations on calcium sensitivity.

To test our hypothesis that the phosphorylation of additional TnI Tyrosine residues enhances thin filament deactivation to a similar degree as the TnI Tyr-26 residue we measured calcium dissociation from TnC in the thin filament. Our results demonstrated that while all mouse and human tyrosine residues tested decreased calcium sensitivity, only the human TnI tyrosine pseudo-phosphorylation of residues 26 and 29 accelerates Ca^{2+} dissociation from TnC while the mouse TnI tyrosine phosphorylation of residue 134 did not. We propose that because human and mouse TnI tyrosine phosphorylation decreases Ca^{2+} sensitivity in reconstituted thin filaments to similar degrees, the mouse can be used as a model for tyrosine TnI phosphorylation calcium-sensitivity functional effects in the human heart. We also propose that because the mouse TnI tyrosine phosphorylation of residue 134 does not alter calcium dissociation and human TnI tyrosine phosphorylation or residues 26 or 29 do, there is a differential effect of the specific tyrosine residue phosphorylated on the rate of calcium dissociation from TnC. These findings suggest that the similar sensitivity change between human and mouse tyrosine phosphorylation, but difference in dissociation effects, support species specific changes in the thin filament mechanisms that control calcium dissociation and potentially cardiac muscle relaxation.

Our last study focusing on Tyr-134 in mouse cardiac TnI found that the phosphorylation of residue 134 has a decreased calcium-binding sensitivity and an unaltered calcium dissociation from TnC. Unlike the mouse, the corresponding residue in human TnI is a non-phosphorylatable phenylalanine. We predicted this reside in TnI was

altered due to specific functional effects required for human cardiac contraction and relaxation compared to the faster heart rate of the mouse. We believe this site was either evolutionarily gained in the mouse for this ability or evolutionarily lost in the human for it was not needed. The evolution loss or gain of this phosphorylatable tyrosine residue in human cardiac TnI may therefore be important to human heart contraction and the re-introduction of the phosphorylatable tyrosine may serve as a target to modulate cardiac contractile properties.

Chapter 6-Future Directions and Conclusion

Future Direction

Additional studies are needed to extend the results obtained from these experiments. Calcium-binding experiments are needed to analyze the additional phosphorylatable tyrosine residue on the human TnI, Tyr-112, to determine if the phosphorylation at this site has similar functional effects observed to the results seen from this study for the phosphorylation of the Tyr-26 and Tyr-29 sites. Calcium-binding experiments are also needed to analyze the effect of the phosphorylation of human TnI Phe-133 by mutation to tyrosine and it phosphorylation and comparison of its functional effects to those of the mouse TnI Tyr-134 residue when phosphorylated. Such experiments would support the need to investigate targeted phosphorylation of additional human TnI tyrosine residues to modulate cardiac contraction in the future.

Furthermore, future studies should consider calcium sensitivity of the thin filament containing a combination of phosphorylated tyrosine sites as they would likely occur in the heart. Incorporating multiple pseudo-phosphorylations has the potential to nullify or amplify the effects of a single tyrosine residue phosphorylation so it would be important to assess the crosstalk of multiple phosphorylated sites present in human and mouse TnI.

Lastly, the experiments conducted in this report were all conducted in vitro to determine if there was a functional effect. Prospective studies should be conducted in vivo experimentation to determine effect of TnI phosphorylation on cardiac myocyte contraction rates on basal and diseased hearts.

Conclusion

In the data reported here, we demonstrate human TnI tyrosine phosphorylation functions similarly to the phosphorylation of mouse TnI tyrosine residues, resulting in decrease Ca^{2+} sensitivity and accelerated dissociation in the reconstituted thin filament. In addition, we demonstrate the unique Tyr-134 residue in mouse TnI exhibits decreased calcium sensitivity of the thin filament without altering calcium dissociation.. These effects of thin filament phosphorylation of TnI tyrosine residues to alter myofilament regulation and their potential effect on cardiac contractile function present a potentially novel pathway to modulate heart function.

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Figure legends

Fig 1. **SDS-PAGE gel demonstrating purity of expressed proteins.** HcTnT; purified human cardiac TnT, HcTnI WT; purified human cardiac WT TnI, HcTnI Y26D; purified human cardiac TnI Y26D, HcTnI Y26E; purified human cardiac TnI Y26E, HcTnI Y29E; purified human cardiac TnI Y29E, and HcTnC; purified human cardiac TnC.

Fig. 2 **Steady-state thin filament calcium binding of human TnI.** Change in IAANS fluorescence resulting from Ca^{2+} binding to TnC in thin filaments containing non-phosphorylated human wild-type TnI (Hc*TnI WT, grey circle*), pseudo-phosphorylated human Tyr-26 to Asp TnI (HcTnI Y26D*, red circle*), pseudo-phosphorylated human Tyr-26 to Glu TnI (*HcTnI Y26E, black circle*) and pseudo-phosphorylated human Tyr-29 to Glu TnI (*HcTnI Y29E, pink)*.

Fig 3. **Average change in IAANS fluorescence of isolated human troponin by stopped-flow calcium dissociation**. Human TnI (Wt, Y26D, Y26E, Y29E). Pseudo-phosphorylation shows no significant influence on calcium-dissociation from troponin.

Fig 4. **Average change in IAANS fluorescence in human thin filaments by stopped-flow calcium dissociation**. Human troponin in reconstituted thin filament (WT, Y26D, Y26E, Y29E). Pseudo-phosphorylation shows significant influence on calcium-dissociation from Troponin.

Fig. 5 **SDS- PAGE gel demonstrating purity of expressed proteins.** RcTnI; purified rat cardiac TnI, RcTnT; purified rat cardiac TnT and RcTnC; purified rat cardiac TnC.

Fig 6. **SDS-PAGE gel demonstrating purity of expressed proteins**. McTnT; purified mouse cardiac TnT, McTnI WT; purified mouse cardiac WT TnI, McTnI Y26E; purified mouse cardiac TnI Y26E, McTnI Y134E; purified mouse cardiac TnI Y134E, McTnC; and purified mouse cardiac TnC.

Fig 7. **Steady-state thin filament calcium binding of human, mouse and rat TnI.** Change in IAANS fluorescence resulting from Ca^{2+} binding to TnC in thin filaments containing non-phosphorylated human wild-type TnI (Hc*TnI WT, grey circle*), non-phosphorylated mouse wild-type TnI (*McTnI WT, red circle*) and non-phosphorylated rat wild-type TnI (*RcTnI, black circle*).

Fig. 8 **Representative change in IAANS fluorescence of human thin filaments by stopped flow calcium dissociation.** Thin filaments containing Human Wt, Human Y29E and Human Y26E. Traces are staggered for clarity.

Fig. 9 **Representative change in IAANS fluorescence of mouse thin filaments by stopped flow calcium dissociation.** Thin filaments containing Mouse Wt and Mouse Y134E. Traces are staggered for clarity.

Fig. 10 **Steady-state thin filament calcium binding of mouse WT and pseudo-phosphorylated TnI.** Change in IAANS fluorescence resulting from Ca^{2+} binding to TnC in thin filaments containing non-phosphorylated mouse wild-type TnI (Mc*TnI WT,red circle*), pseudo-phosphorylated mouse Tyr-26 to Glu TnI (McTnI Y26E, *grey circle*) and pseudo-phosphorylated mouse Tyr-134 to Glu TnI (*McTnI Y134E, black circle*).

Fig 11. **Average change in IAANS fluorescence in mouse thin filament by stopped-flow calcium dissociation**. Mouse troponin in reconstituted thin filament (WT, Y134E). Pseudo-phosphorylation shows significant influence on calcium-dissociation from Troponin.

Fig 12. **Calcium required to reach 50% maximal activation (pCa_{50}) in steady-state calcium-binding titrations.** McTnWt (n=19), McTnY26E (n=14) and McTnY134E (n=7).

Fig 13. **Average change in IAANS fluorescence of mouse isolated troponin by stopped-flow calcium dissociation**. Mouse TnI (Wt, Y26E, Y134E). Pseudo-phosphorylation shows no significant influence on calcium-dissociation from troponin.

Fig 14. **Calcium required to reach 50% maximal activation (pCa_{50}) in steady-state calcium-binding titrations.** HcTnWT (n=14), HcTnY26D (n=3), HcTnY26E (n=6) and HcTnY29E (n=3).

Figure 1.

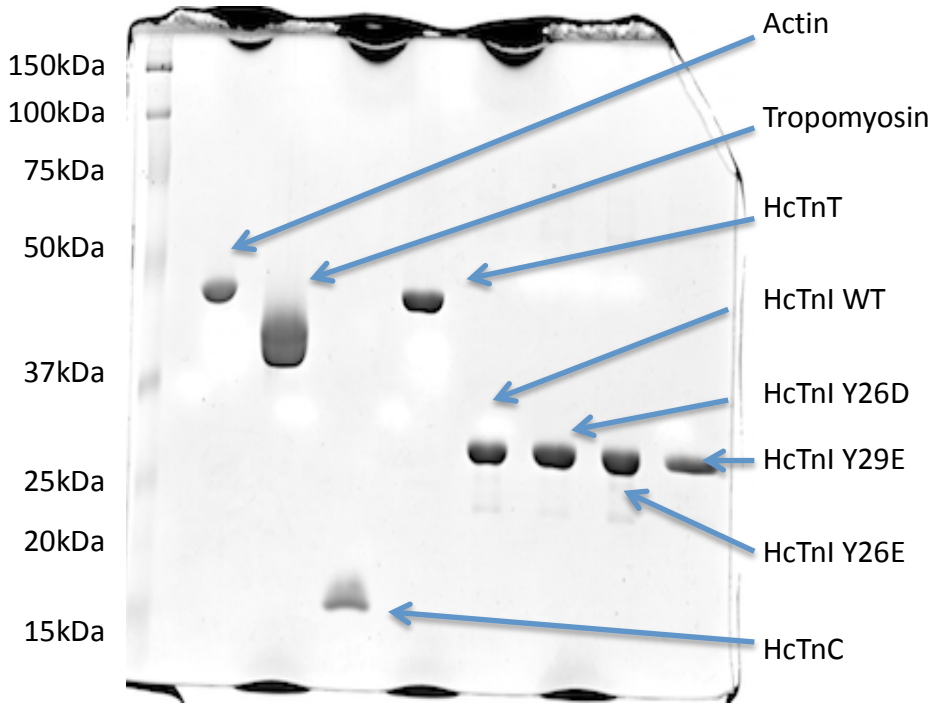
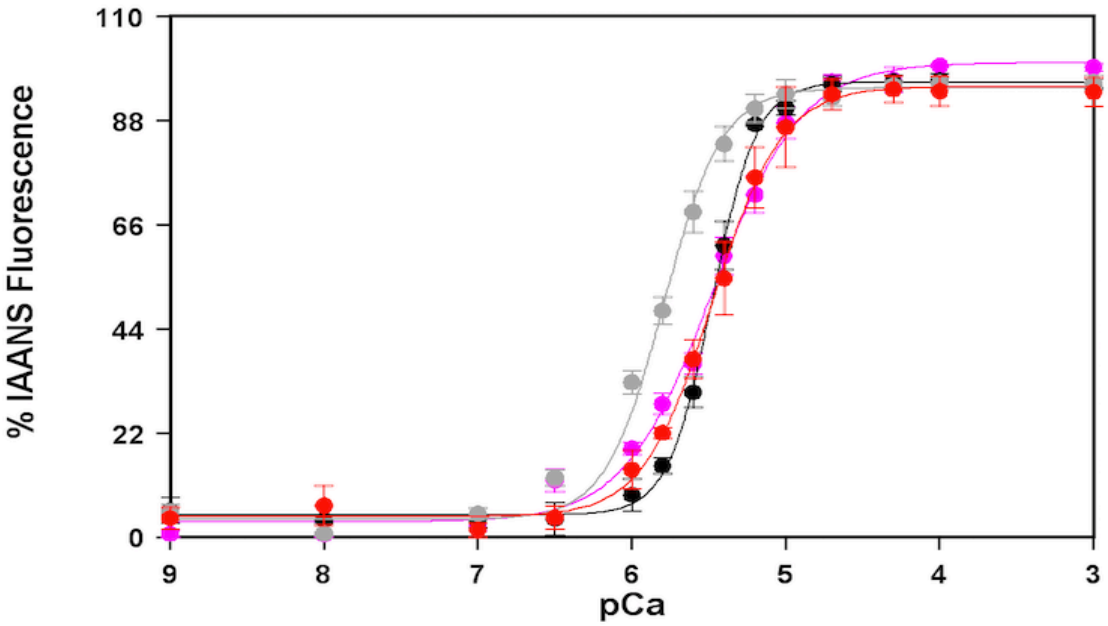

150kDa
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa
Actin
Tropomyosin
HcTnT
HcTnI WT
HcTnI Y26D
HcTnI Y29E
HcTnI Y26E
HcTnC

Figure 2.



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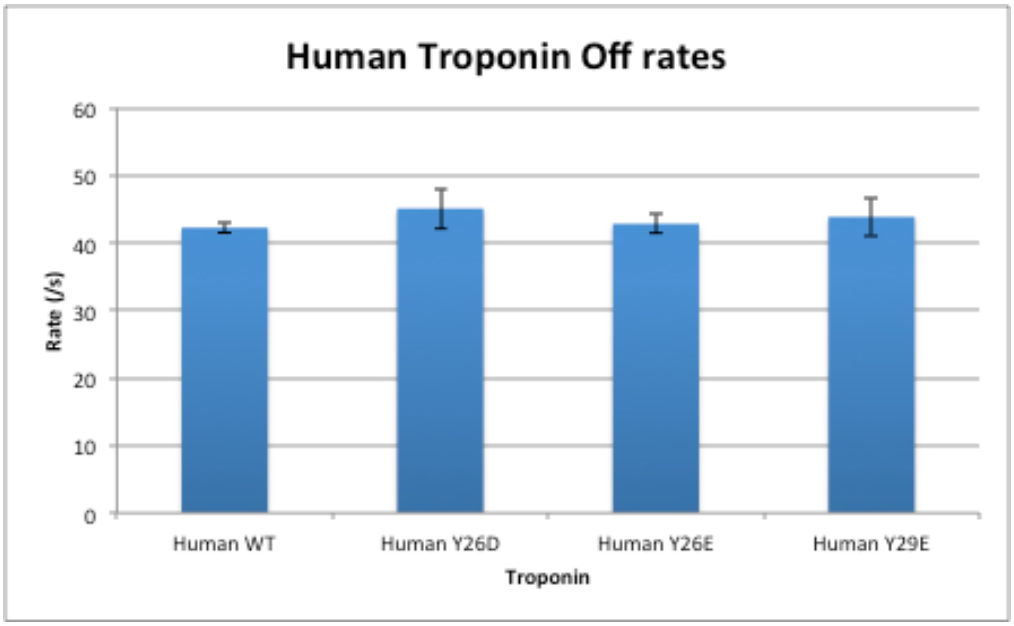

Human Troponin Off rates
60
50
40
30
20
10
0
Rate (/s)
Human WT
Human Y26D
Human Y26E
Human Y29E
Troponin

Figure 4.

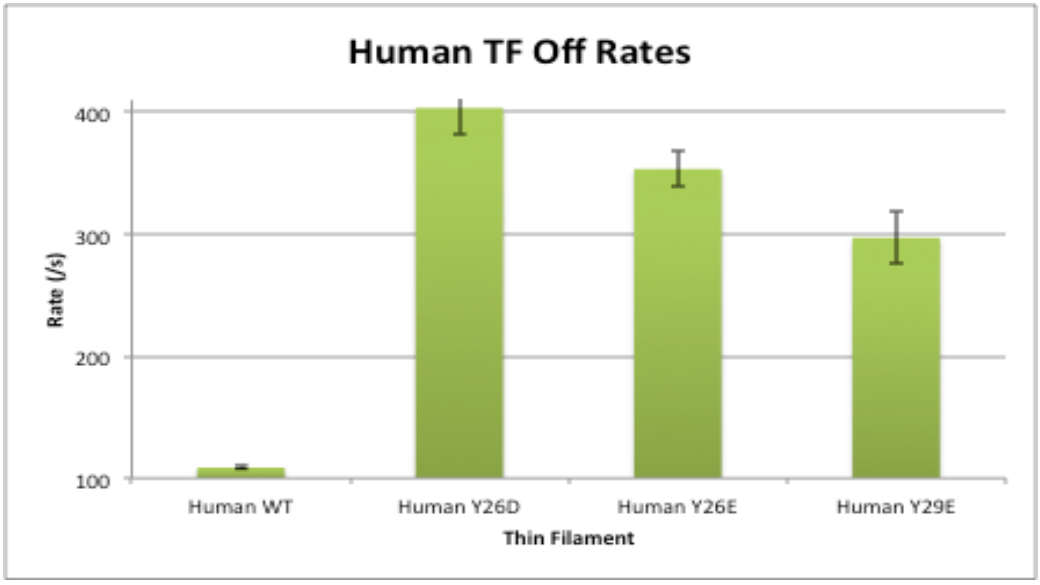

Human TF Off Rates
400
300
200
100
Rate (/s)
Human WT
Human Y26D
Human Y26E
Human Y29E
Thin Filament

Figure 5.

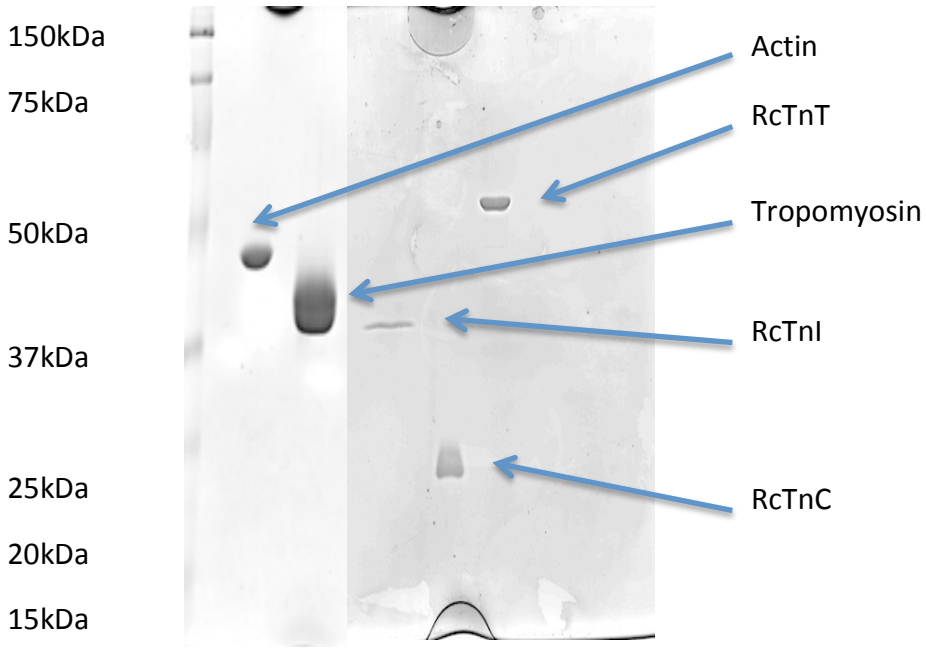

150kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa
Actin
RcTnT
Tropomyosin
RcTnI
RcTnC

Figure 6.

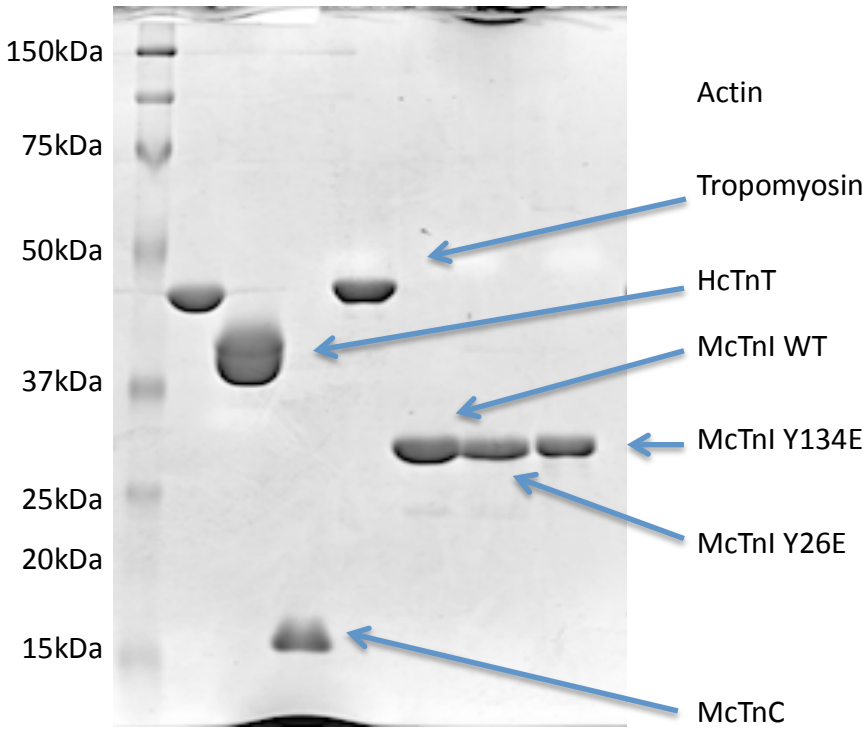
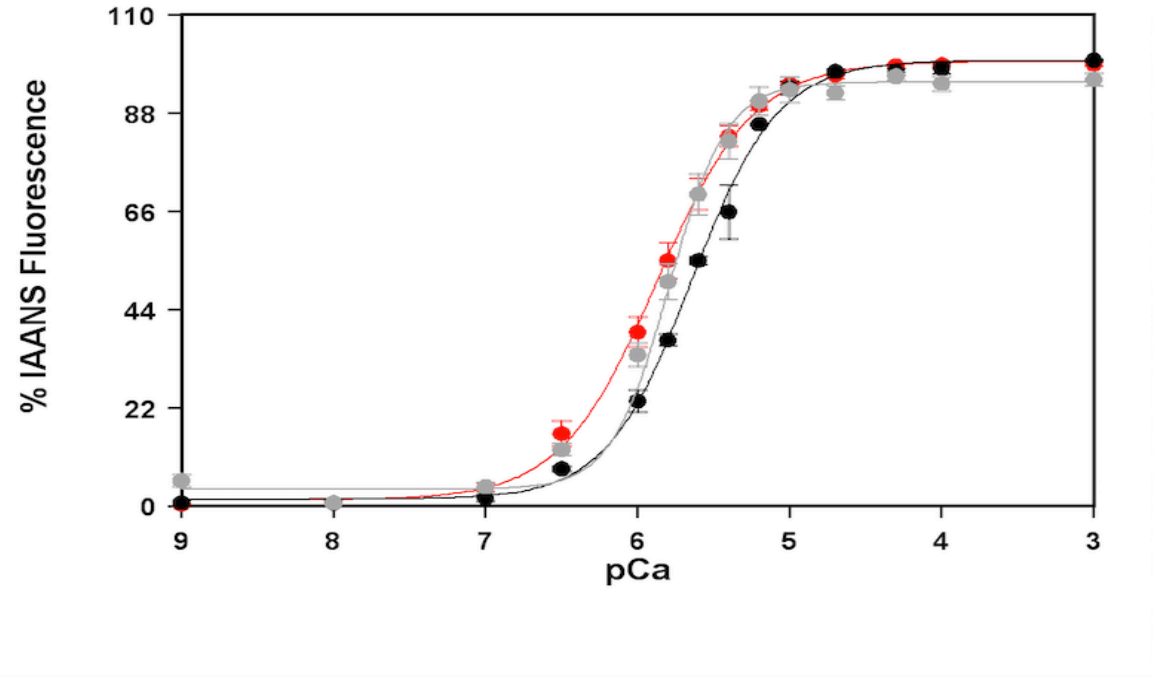

150kDa
75kDa
50kDa
37kDa
25kDa
20kDa
15kDa
Actin
Tropomyosin
HcTnT
McTnI WT
McTnI Y134E
McTnI Y26E
McTnC

Figure 7.



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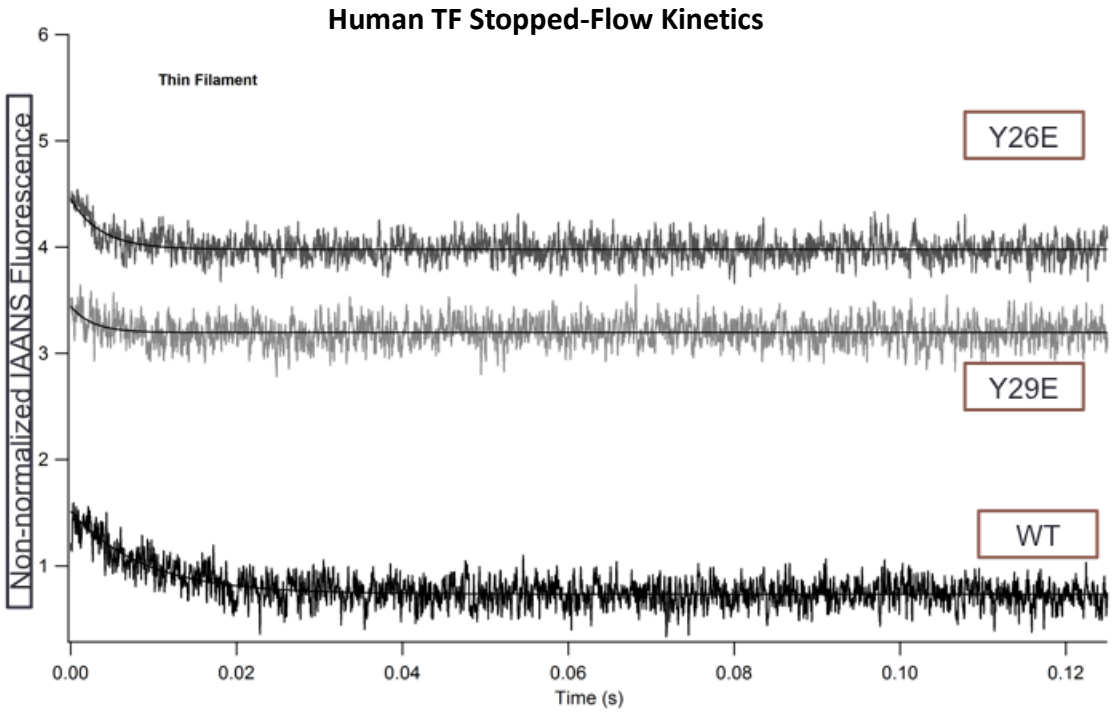

Human TF Stopped-Flow Kinetics
Thin Filament
Non-normalized IAANS Fluorescence
Y26E
Y29E
WT
6
5
4
3
2
1
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Time (s)

Figure 9.

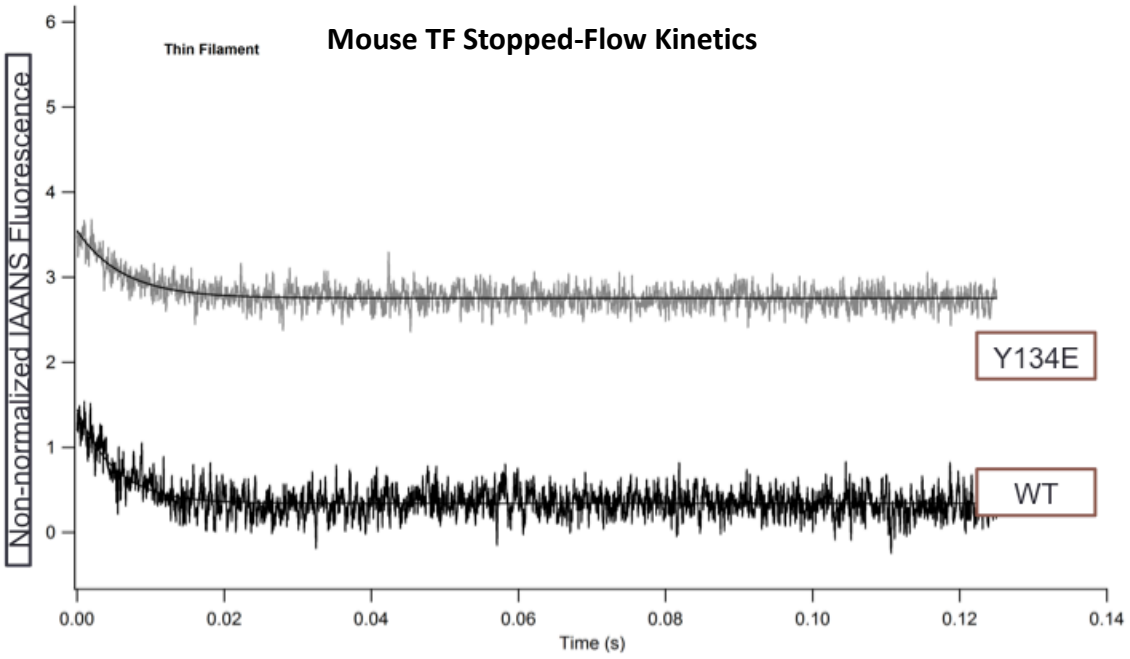

Thin Filament
Mouse TF Stopped-Flow Kinetics
Non-normalized IAANS Fluorescence
Y134E
WT
Time (s)
0
1
2
3
4
5
6
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14

Figure 10.

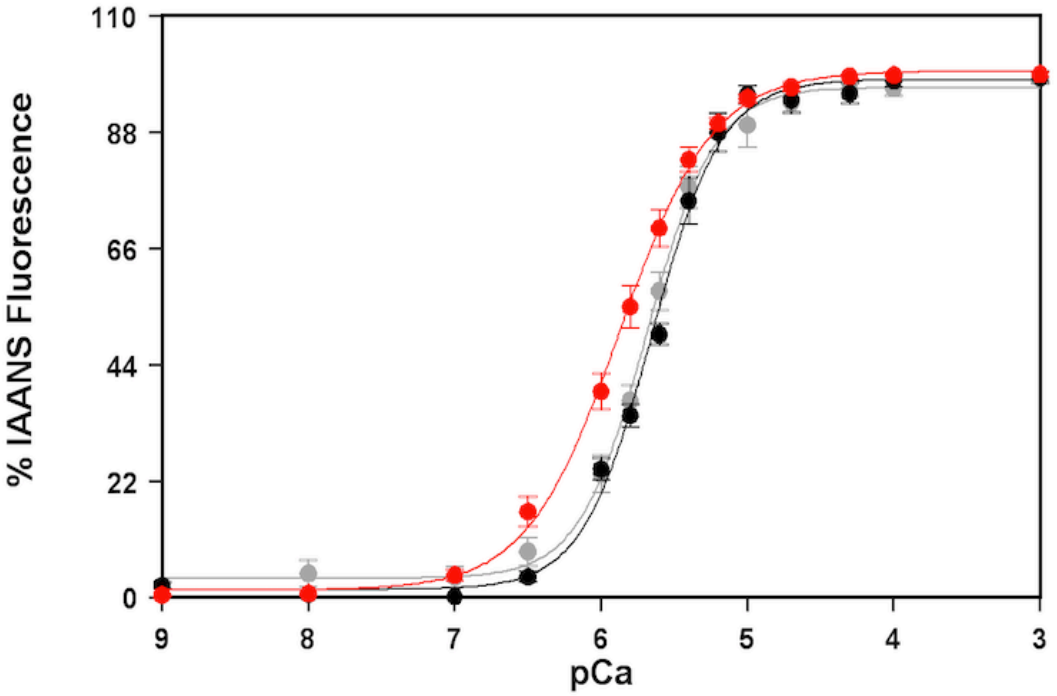


Figure 11.

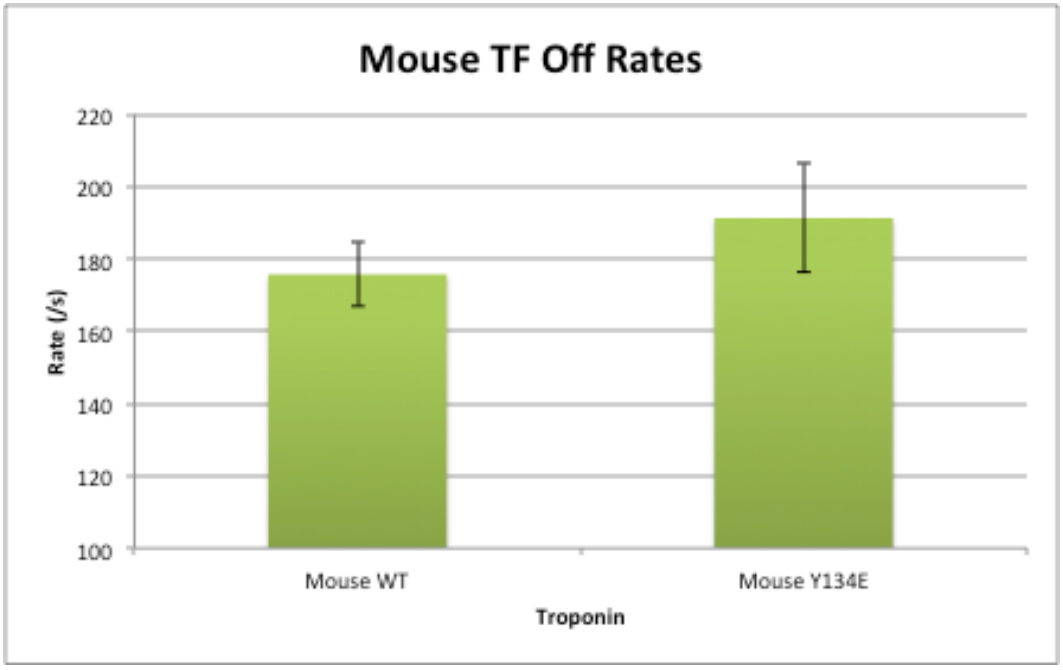

Mouse TF Off Rates
220
200
180
160
140
120
100
Rate (/s)
Mouse WT
Mouse Y134E
Troponin

Figure 12.

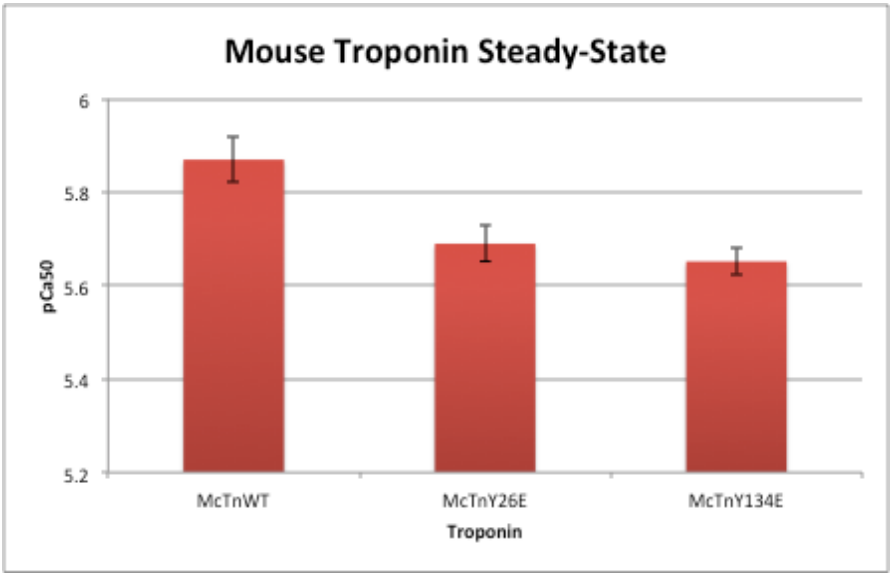

Mouse Troponin Steady-State
6
5.8
5.6
5.4
5.2
pCa50
McTnWT
McTnY26E
McTnY134E
Troponin

Figure 13.

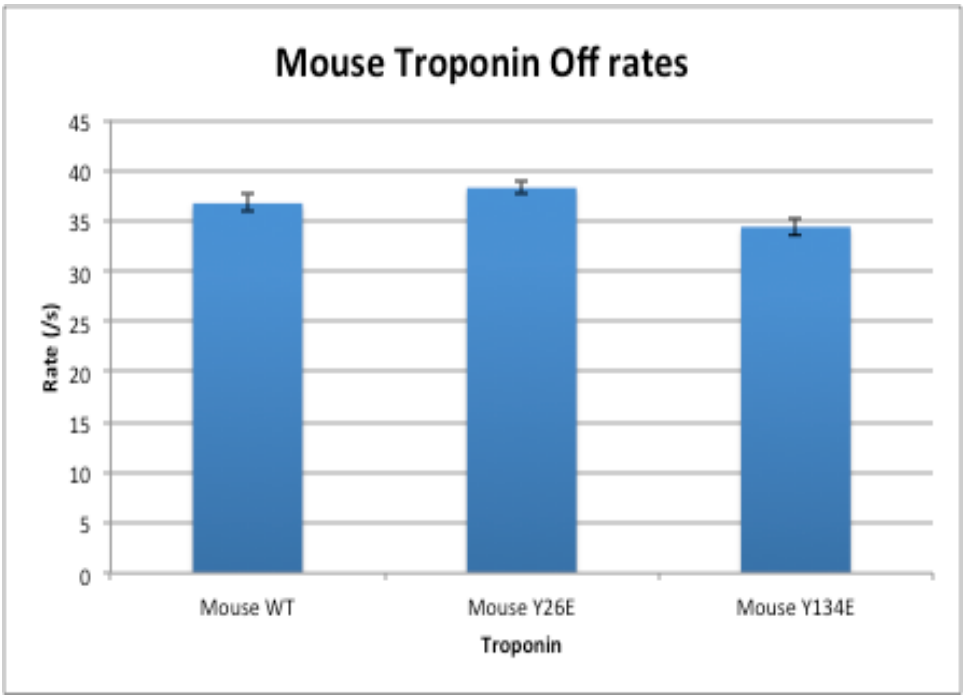
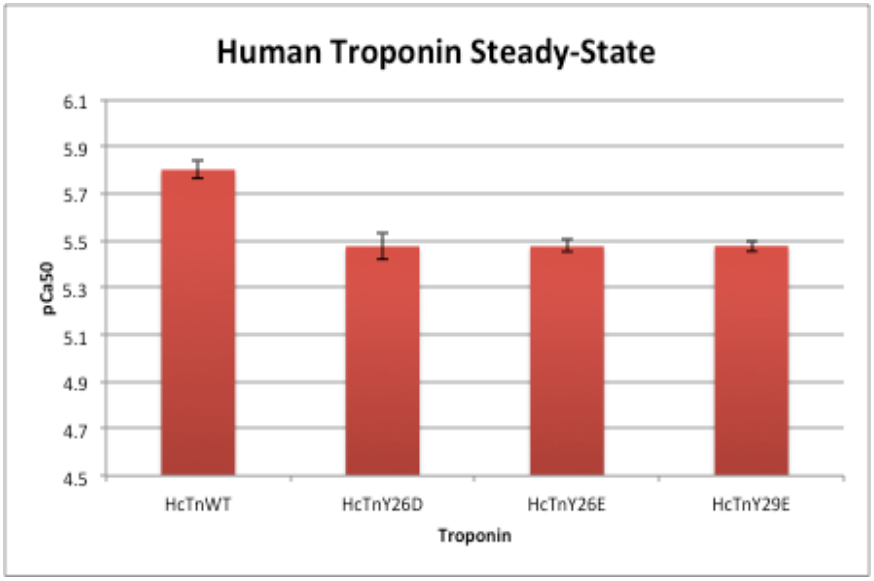

Mouse Troponin Off rates
45
40
35
30
25
20
15
10
5
0
Rate (/s)
Mouse WT
Mouse Y26E
Mouse Y134E
Troponin

Figure 14.


Human Troponin Steady-State
6.1
5.9
5.7
5.5
5.3
5.1
4.9
4.7
4.5
pCa50
HcTnWT
HcTnY26D
HcTnY26E
HcTnY29E
Troponin